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Optimization of experimental design parameters for high-throughput chromatin immunoprecipitation studies

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ABSTRACT

High-throughput, microarray-based chromatin immunoprecipitation (ChIP-chip) technology allows *in vivo* elucidation of transcriptional networks. However this complex is not yet readily accessible, in part because its many parameters have not been systematically evaluated and optimized. We address this gap by systematically assessing experimental-design parameters including antibody purity, dye-bias, array-batch, inter-day hybridization bias, amplification method and choice of hybridization control. The combined performance of these optimized parameters shows a 90% validation rate in ChIP-chip analysis of Myc genomic binding in HL60 cells using two different microarray platforms. Increased sensitivity and decreased noise in ChIP-chip assays will enable wider use of this methodology to accurately and affordably elucidate transcriptional networks.

INTRODUCTION

The combination of chromatin immunoprecipitation (ChIP) and high-throughput DNA microarray technology (chip) provides a powerful method (ChIP-chip) for mapping protein–DNA interactions *in vivo*. The ChIP-chip procedure was first used to study yeast transcription factors (1–4) and has recently been exploited for similar studies in mammalian cells (5–15). In the ChIP-chip procedure, genomic DNA is precipitated using specific and control antibodies, then labeled and hybridized to

genomic microarrays. Several different array platforms have been used, including proximal promoter region arrays (11), CpG island arrays (12,15) and whole-genome tiling arrays (16,17). The technique is extremely versatile and can identify target genes whose regulatory regions are bound by transcription factors through both protein–DNA and protein–protein interactions. ChIP-chip can be applied to any cell or tissue, making it possible to decode gene regulatory networks *in vivo*. Certainly, the ability to profile *bona fide* target genes has been revolutionized by ChIP-chip. Unlike mRNA expression analysis, the genetic program specifically directed by the transcription factor can be distinguished from subsequent downstream regulatory events (18–22).

Although ChIP-chip has become the standard for discovering the genomic binding sites of transcriptional regulators there is wide variability in experimental design (23). This variability has complicated and delayed widespread application, and is a reflection of the large number of parameters that must be carefully optimized in ChIP-chip experiments. For example, the number of cells or amount of tissue used as starting material varies widely from one study to another (24,25). The protein–protein and/or protein–DNA cross-linking, chromatin sonication, as well as antibody sensitivity and purity characteristics can also vary significantly. In most studies, the enriched DNA recovered after the ChIP procedure is amplified. A variety of amplification methods have been developed, including ligation-mediated PCR (25,26), random primed PCR (27), T7 primed PCR (28) and Whole Genome Amplification (WGA) (29), and it is unclear which method is most appropriate for ChIP studies. Finally, when the amplified and labeled DNA

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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is hybridized to a microarray a control sample must be selected and the effects of array batch and dye-swap status considered.

Experimental design parameters for mRNA expression arrays have been extensively evaluated by a number of groups over the past decade (30–36). As a result, the key factors are well understood and the assay has been optimized. It is possible, for example, to estimate the number of biological replicates required to sufficiently power a specific hypothesis-testing question (37). Despite this clear evidence that parameter optimization can greatly improve the quantity and quality of information retrieved from an array analysis, ChIP-chip design parameters have not yet been thoroughly and systematically investigated, and it cannot be assumed that parameters and processes would be the same for both mRNA and ChIP-chip arrays.

Here, we fill that gap by providing a comprehensive evaluation of experimental design parameters for ChIP-chip studies. Through a series of validation studies we address both the parameters previously investigated for mRNA expression studies as well as those specific to ChIP-chip experiments. We exploit a well-characterized system: the genomic binding of the Myc oncoprotein in HL60 cells, a human myelogenous leukemia cell line, combined with CpG island arrays (38). Many parameters for successful ChIP-chip studies were analyzed, including antibody purity, array batch variability, dye-bias, inter-day hybridization-variability, amplification procedure and hybridization control. In addition, we evaluated the combined effect of the optimized parameters by conducting a Myc ChIP-chip study using an alternative oligonucleotide array platform. Our results show a high rate of validation by real time Q-PCR. The raw data from this study, encompassing over 100 arrays has been deposited in the Gene Expression Omnibus (GEO) repository at NCBI. Our careful description of ChIP-chip experimental design is a key step towards enabling widespread use of this important technology for the rapid elucidation of global transcriptional regulatory networks.

MATERIALS AND METHODS

Antibody production and purification

The DNA fragment corresponding to the Myc 1-262 N-terminal domain polypeptide was cloned into pET15b vector (Novagen 69661-3) at 5'-NdeI and 3'-BamHI sites. His-c-Myc (1–262) fusion protein was purified under denatured conditions using Talon beads (BD Biosciences, Mississauga, ON, Canada). His-c-Myc (1–262) fusion protein was purified under denatured conditions using Talon beads (BD Biosciences) as follows, the cell pellet was homogenized in 40 ml of lysis buffer pH 8.0 (5 mM imidazole, 20 mM Tris, 500 mM NaCl, 10 μ M ZnCl₂, 6 M Guanidine hydrochloride, 0.1% Triton X-100, 1 mM β -mercaptoethanol) and sonicated three times for 3 min at duty cycle 30 and 30% output. The lysate was then centrifuged at 14 000 r.p.m. for 30 min at 4°C. Next, 4 ml of 50% Talon beads were washed with 50 ml of binding buffer pH 8.0 (lysis buffer without TX-100) and

pelleted at 1800 r.p.m. for 5 min. The lysed supernatant was incubated with washed Talon beads with gentle swirling for 1 h at room temperature and then centrifuged at 1800 r.p.m. for 5 min at 4°C. The beads were washed once with binding buffer and once with washing buffer pH 8.0 (Binding buffer with 10 mM imidazole). The proteins were eluted by adding 1 ml of elution buffer pH 8.0 (binding buffer with 500 mM imidazole) to the beads and after centrifugation at 1800 r.p.m. for 5 min at 4°C the supernatant was collected. The protein concentration is checked and the elution step is repeated several times until no more or very low protein is detected. The purity of the protein sample is then checked by SDS-PAGE. The denatured protein sample was precipitated by adding 900 μ l of 95% ethanol to 100 μ l of protein sample, and then centrifuged at 14 000 r.p.m. for 10 min. The pellet was resuspended in 20 μ l of SDS-loading buffer before loading. The purified protein was concentrated to 2.8 mg/ml before injecting into the rabbit. The purified protein was then dissolved in a volume of 500 μ l of PBS per rabbit. Primary immunizations were done with 500 μ g of purified protein per rabbit. First boost was done with 250 μ g of purified protein. Two subsequent boosts were done with 50 μ g of purified protein. The final bleed was then purified using Enchant IgG Purification Kit with Protein A, IgG Purification (Pall 5300-IGGPROA) as follows: the sample was diluted 1:1 with binding buffer and applied to the equilibrated Protein A affinity column. The column was washed once with the Protein A Binding Buffer and then eluted with the Protein A Elution Buffer to recover the bound IgG. Each microliter of purified fraction was then neutralized by adding 50 μ l of 1 M Tris, pH 9.5.

Cell culture conditions

HL60 (ATCC) cells were maintained in α -MEM with 10% FBS.

Chromatin immunoprecipitation

Exponentially growing HL60 cells were cross-linked with 1% formaldehyde for 10 min at 37°C. The cross-linking reaction was quenched by addition of glycine to a final concentration of 0.125 M for 5 min, followed by two washes with phosphate-buffered saline (PBS). Cells were resuspended in cell lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% [v/v] NP40, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin) for 10 min on ice and then pelleted (5000 r.p.m., 5 min, 4°C). The pellet was resuspended in 1 ml of nuclei lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin) for 10 min on ice and then sonicated using 8 pulses (12–13 Watts, setting 10, 10 s per pulse, 45 s on ice between pulses) from a Model 60 Sonic Dismembrator (Fisher Scientific 15-338-53) to generate fragments between 600 bp and 1000 bp. Lysates were centrifuged for 10 min at 21 000g at 4°C. Supernatants were diluted into an equal volume with IP dilution buffer (0.01% SDS, 1.1% Triton-X100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 0.2% Sarkosyl, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin) and precleared for 30 min at 4°C with protein G-PLUS

agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-2002). Prior to use, G-PLUS agarose beads were blocked with salmon sperm DNA at a final concentration of 50 µg/ml and rotated overnight at 4°C. Diluted and cleared extracts corresponding to 10×10^6 HL60 cells were incubated and rotated at 4°C for ~12–16 h with each of the following antibodies: no-antibody control, 0.7 µg normal rabbit IgG (Santa Cruz Biotechnology sc-2027), 0.7 µg N262 (Santa Cruz Biotechnology sc-764), for the N262 home-made unpurified and purified antibodies, we determined empirically, by serial dilutions, the amount of antibody to be used. A 50 µl of salmon sperm DNA pre-blocked Protein G-PLUS agarose beads were added to each sample, incubated on a rotating platform at 4°C for 3 h. Each pellet was washed once with 1.4 ml of sonication buffer and then twice with 1.4 ml of high salt buffer (0.1% [v/v] SDS, 1% [v/v] Triton X-100, 1 mM EDTA, 50 mM HEPES, 500 mM NaCl, 0.1% [w/v] sodium deoxycholate) and then once with 1.4 ml LiCl Buffer (250 mM LiCl, 1% [v/v] NP-40, 1% [w/v] sodium deoxycholate, 1 mM EDTA, 1 mM Tris pH 8) and finally twice with 1.4 ml TE pH 8 (10 mM Tris pH8, 1 mM EDTA). For each wash, the pellets were mixed for 5 min at room temperature then pelleted (3000 r.p.m., 30 s, room temperature). After the last wash, the pellets were eluted in 300 µl of Elution buffer (1% [w/v] SDS, 10 mM Tris pH 8, 5 mM EDTA), incubated at 65°C for 15 min, and then pelleted (3000 r.p.m., 3 min, room temperature). Cross-links were reversed in the presence of 200 mM NaCl at 65°C over-night and samples were treated with RNase A (Sigma R5500). After ethanol precipitation, the samples were resuspended in 100 µl of TE (10 mM Tris, pH 7.5, 1 mM EDTA), 25 µl of 5× proteinase K buffer (1.25% SDS, 50 mM Tris, pH 7.5, 25 mM EDTA), and 1.5 µl of proteinase K (Roche 1413783) and incubated at 42°C for 2 h. DNA was extracted from each sample using phenol:chloroform:isoamyl alcohol (25:24:1), then precipitated with 1/10th volume of 3 M sodium acetate (pH 5.3), 5 µg of glycogen, and 2 volumes of ethanol at –20°C overnight. Pellets were collected by microcentrifugation and resuspended in 60 µl of H₂O.

PCR

A total of 2 µl of the ChIP DNA was PCR amplified using promoter-specific primers. All PCR primers (synthesized by Sigma Genosys, Oakville, ON, Canada or Invitrogen, Burlington, ON, Canada) were resuspended in sterile RNase-/DNase-free water. In general, each 10 µl PCR reaction, set up at room temperature, was composed of 10 ng/µl of each primer (fwd/rev), 1× PCR buffer, 0.2 mM dNTP mix, sterile water, DNA template and 0.2 µl HotstarTaq DNA polymerase (Qiagen, Mississauga, ON, Canada, 203205); the HotstarTaq was activated by initiating the PCR program at 95°C for 15 min. Amplification of each PCR primer set was performed using multiple annealing temperatures along a 52–68°C gradient for 30 s. Primer sequences are provided in Supplementary Table 8. All PCR reactions were electrophoresed on 100 ml 1.2% agarose gels containing 4 µl of 10 mg/ml EtBr, and then visualized by UV fluorescence.

Real-time quantitative PCR

Real-time quantitative PCR (Q-PCR) amplification was conducted using the SYBR Green assay in the ABI PRISM 7900-HT (Applied Biosystems, Foster City, CA, USA). Each 12 µl quantitative PCR reaction was composed of the following: 2 µl of the DNA isolated in the ChIP, 1× PCR Buffer, 2.5 mM MgCl₂, 0.17 mM dNTP mix, sterile water, 0.25× SYBR Green (Sigma S9430), 0.2 µl Rox reference dye (Invitrogen 12223-012) and 0.05 µl Platinum Taq DNA polymerase (Invitrogen 10966-034) and was performed in triplicate in a 384-well plate. The reactions began at 50°C for 2 min and then were activated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Human male genomic DNA (Novagen 70572) was used as standard real-time Q-PCR was conducted in triplicates for each of the three independent biological replicates. The Q-PCR data were analyzed by calculating the Myc/IgG ratios for each target gene, and the Chr6 negative control. A symmetrical distribution was then obtained by log₂-transformation of each individual ratio. Statistical testing involved comparison of the ratios for each target gene to the ratios of the Chr6 negative control using a two-tailed paired *t*-test. Primer sequences are provided in Supplementary Table 8. The primers were designed using Primer Express Software Version 2.0 (Applied Biosciences) with the following parameters: *T*_m range of 58–60°C, the primer length between 19 and 50 bp, optimal primer length of 20 bases, and amplicon length of 80–200 bp.

Random primer PCR amplification methods

The immunoprecipitated DNA was amplified as described elsewhere (www.microarrays.ca/support/PDFs/ChIP_Protocol_for_Microarray_Analysis_Beads_corr_01-12-06.pdf) with the following modifications: 15 µl of Round A are mixed with Round B1 Reaction Mix to a final volume of 100 µl [4 µl of 50 mM MgCl₂, 10 µl of 10× PCR Buffer, 2 µl of 10 mM dNTPs, 2.5 µl of 100 µM of Primer B (5′GTTTCCCAGTCACGATC3′), 1 µl of Taq DNA Polymerase (Promega, Madison, WI, USA, M1861)]. Amplification/Nucleotide Incorporation Program: (94°C for 30 s, 40°C for 30 s, 50°C for 30 s, 72°C for 1 min) × 20 cycles, 72°C for 2 min.

A total of 15 µl of Round B1 were mixed with Round B2 Reaction Mix to a final volume of 50 µl [2 µl of 50 mM MgCl₂, 5 µl of 10× PCR Buffer, 0.5 µl of dNTP Mix B2 (25 mM of dATP, dCTP, dGTP, 12.5 mM dTTP), 1.5 µl of 2 mM aa-dUTP, 1 µl of 100 µM of Primer B (5′GTTTCCCAGTCACGATC3′), 1 µl of Taq DNA Polymerase (Promega M1861)]. Amplification/Nucleotide Incorporation Program: (92°C for 30 s, 40°C for 30 s, 50°C for 30 s, 72°C for 1 min) × 15 cycles, 72°C for 2 min. Round B2 amplified DNA was concentrated using Microcon columns (Millipore, Billerica, MA, USA, Microcon YM-30). Once the ChIP DNA was amplified to 3 µg, PCR reactions were performed with 10 ng amplified DNA and were identical to the reactions performed after the ChIP DNA was initially isolated. This PCR was used to determine whether specificity between the IgG or NoAb and antibody samples was maintained after the amplification.

Ligation-mediated PCR amplification

The DNA from each ChIP reaction was treated as described elsewhere (26). The PCR was performed using the following cycling protocol: 1 cycle of 55°C for 2 min, 72°C for 5 min, and 95°C for 2 min and the corresponding number of cycles for ligation-mediated PCR (LM-PCR) A and LM-PCR B (see below LM-PCR A and B cycle conditions) at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, ending with 4 min at 72°C and then an endless cooling at 4°C. After the PCR, the DNA was again isolated using the QIAquick PCR purification kit (Qiagen 28106). The concentration of the purified DNA was determined on the NanoDrop ND-1000 spectrophotometer. Often the 3 µg required for hybridization was not acquired after this first round of amplification, so another round of amplification would be performed, followed by purification and determination of the DNA concentration as previously described. LM-PCR A amplification: 20 cycles followed by QIAquick PCR purification, and then 15 cycles followed by QIAquick PCR purification. LM-PCR B amplification: 20 cycles followed by QIAquick PCR purification, repeated three rounds, sequentially. Once the ChIP DNA was amplified to 3 µg, PCR reactions of positive and negative control genes (Supplementary Table 8) were performed with 10 ng amplified DNA and were identical to the reactions performed after the ChIP DNA was initially isolated. This PCR was necessary to determine whether specificity between the IgG or NoAb and antibody samples was maintained after the amplification.

Labeling reaction

A total of 3 µg of amplified DNA from the antibody sample, no antibody, or IgG sample were vacuum desiccated and resuspended in 2.5 µl of H₂O (Sigma W4502). 4.5 µl of 0.1 M NaHCO₃ at pH 9 was added. The DNA was resuspended by vortexing several times. 2 µl of Cy3 or Cy5 dye (Amersham, PA23001 and PA25001, respectively) were added to the sample. The mix was incubated for 1.5 h at room temperature in the dark. After incubation, 35 µl of 100 mM Na-Acetate pH5.2 was added and topped with H₂O (Sigma W4502) to a total volume of 100 µl for each sample. The fluorescent-labeled probes were purified using QIAquick PCR Purification kit (Qiagen 28106). The DNA was eluted with 50 µl of EB buffer from the kit, heated to 65°C. The 100 µl combined ChIP and no antibody samples were vacuum desiccated using a SpeedVac for 60 min at maximum heat.

DNA microarray hybridization

The mixed DNA was resuspended in 5 µl of H₂O and mixed with 85 µl hybridization mix [100 µl of DIG Easy Hyb solution (Roche 603 558), 5 µl of 10 mg/ml calf thymus DNA (Sigma D8661), 5 µl of 10 mg/ml yeast tRNA (Invitrogen 15401-029)]. The resulting solution was incubated at 65°C for 2 min, then cooled to room temperature and applied to a CpG island array slide (UHN Microarray Centre HCGI12K, design is available

at www.microarrays.ca) and incubated overnight at 42°C in a hybridization chamber.

Microarray washing

After hybridization, the microarray slide was washed once with washing buffer 1 (1× SSC, 0.1% SDS) at 50°C for 5 min. This was followed by a wash with washing buffer 1 (room temperature for 5 min) and two washes with washing buffer 2 (0.2× SSC for 5 min) and one final wash with washing buffer 3 (0.1× SSC for 5 min at room temperature). The slide was then dried by spinning at 700 r.p.m. for 15 min. To preserve the Cy5 dye from ozone-dependent degradation, which was observed during the summertime when the hybridization occurred, the slides were then dipped in DyeSaver 2 (Genisphere Q500600) and allowed to air dry.

Microarray scanning

The microarray slides were scanned using a Gene Pix 4000B scanner (Axon Instruments) at multiple PMT voltages.

Validation using agilent promoter arrays

The Chromatin Immunoprecipitation of HL60 cells was performed as described above. In this case, the sonication was performed as follows, 11 pulses (setting high, 26 s per pulse, 26 s on ice between pulses) from a Model Diagenode BioRuptor Sonicator (Diagenode, BioRuptor 200, UCD-200 TM-EX) to generate fragments between 500 and 1000 bp. The immunoprecipitations were performed using 0.7 µg normal rabbit IgG (Santa Cruz Biotechnology sc-2027) or N262 home-made purified antibody as described above. The WGA was performed following a protocol described by O'Geen *et al.* (29). This method efficiently primes the DNA fragments to generate a library of DNA fragments with defined 3' and 5' termini. The library is then replicated using linear amplification in the initial steps followed by a limited round of geometric amplifications. Entire ChIP samples with an average size of 500–1000 bp of DNA fragments were used for the library generation and subsequent amplification. For the library preparation and the amplification (round 1), GenomePlex Complete WGA kit was used (Sigma WGA2) as follows. Library Preparation step: 2 µl of Library Preparation Buffer was added to the ChIP material that has been concentrated to 10 µl. One microliter of Library Stabilization Solution was added and heated at 95°C for 2 min in a thermal cycler and immediately cooled on ice. One microliter of Library Preparation Enzyme was added and incubated in a thermal cycler pre-cooled to 16°C (16°C for 20 min, 24°C for 20 min, 37°C for 20 min, 75°C for 5 min). Amplification step (round 1): the following master mix was added to each sample prepared on the previous step, 7.5 µl of 10× Amplification Master Mix, 47.5 µl of nuclease-free H₂O and 5 µl of WGA DNA Polymerase. Samples were incubated in a thermal cycler (95°C for 3 min, then 14 cycles of 94°C for 15 s, and 65°C for 5 min). Samples were then purified using QIAquick PCR Purification kit (Qiagen 28106). A 10 ng of each amplified sample was tested for

specificity as described above under the PCR title. For the reamplification step (round 2), GenomePlex WGA amplification Kit was used (Sigma WGA3). A master mix of 7.5 μ l of 10 \times Amplification Master Mix, 47.5 μ l of nuclease-free H₂O and 5 μ l of WGA DNA Polymerase was added to 15 ng of purified amplification product previously diluted in nuclease-free H₂O to 10 μ l volume. Samples were then purified using QIAquick PCR Purification kit (Qiagen 28106). Amplified sample (10 ng) was tested for specificity as described above. WGA amplified DNA (4 μ g) was hybridized to Agilent 2x244 promoter arrays at the UHN microarray center (Toronto, Canada). Based on our previous results a single batch of arrays was used with lot number 19585. Arrays were labeled using the Agilent Genomic DNA Labelling Kit and hybridized using the aCGH Hybridization Kit following the ChIP-on-chip v10 protocol as follows: for each 1x244 promoter array, 2 μ g of DNA was brought to 26 μ l final volume. A 5 μ l of Random Primers (supplied with Agilent Genomic DNA Labeling Kit PLUS) was added and the mix was incubated at 95°C for 3 min, then on ice for 5 min. The 31 μ l were mixed with the Labeling Mix to a final volume of 50 μ l (10 μ l of 5 \times Buffer, 5 μ l of 10 \times dNTP, 3 μ l of 1 mM Cyanine 3-UTP or 1 mM Cyanine 5-dUTP, 1 μ l of Exo-Klenow fragment) and incubated at 37°C for 2 h. The enzyme was then inactivated at 65°C for 10 min. The labeled Genomic DNA was cleaned with Microcon columns (Millipore, Microcon YM-30) and eluted with 80.5 μ l of 1 \times TE. A 5 μ g Cy5-labeled and 5 μ g of Cy3-labeled DNAs were combined in a total volume of 158 μ l. The 158 μ l were mixed with 50 μ l of 1 mg/ml of Human Cot-1 DNA, 52 μ l of 10 \times Agilent Blocking Agent, 260 μ l of 2 \times Agilent Hybridization Buffer. Samples were heated at 95°C for 3 min, and then incubated at 37°C for 30 min. A 490 μ l of the sample were applied to the 1x244 promoter array assembled in a chamber and incubated in a rotisserie hybridization oven at 65°C and 20 r.p.m. for 40 h. After hybridization the microarray slide was washed with Oligo aCGH/ChIP-on-chip Wash Buffer for 5 min at room temperature, followed by a wash for 5 min at 31°C. QC metrics used were based on the ChIP-on-chip requirements and no arrays were removed or repeated.

CpG island microarray data preprocessing

Array images were first manually examined for image artifacts, then quantitated using GenePix Pro (v6.0.1.27). A scan that maximized the dynamic range without saturating high-intensity signals was selected and carried forward for analysis. This scan was quantified using the 'circular' segmentation algorithm, then preprocessed using the variance-stabilizing normalization algorithm (VSN). VSN was implemented in the R statistical environment (v2.4.1) in the BioConductor open-source project (39). Version 1.12.0 of the vsn package was used, with print-tip groups used as strata, and default parameterization except for using 1000 iterations for fitting (increased from the default of 10). All raw and processed have been made publicly available in the GEO

repository at NCBI with IDs GSE8447, GSE8448, and GSE8449.

To test for differential hybridization, we used general linear modeling, as implemented in the limma package (v2.9.13) (40) of the BioConductor open-source library (39) for the R statistical environment (v2.4.1). First, a spot-wise linear model was fit separately to each major design parameters: array batch, hybridization date and dye-status. No interaction terms were included. A Bayesian moderation of standard error was employed following model-fitting (40). A Gaussian kernel density was then fit to the effect estimates to compare the relative magnitudes of these design parameters. Next, a spot-wise linear model was fit comparing the hybridizations using a commercially available antibody to those using a locally produced one. The top 100 spots from each analysis were identified, and the pair-wise *P*-values plotted to identify trends in antibody specificity. A similar analysis was performed for purified and nonpurified locally produced antibody hybridizations. All model-fitting again employed the limma package (v2.9.13). To compare different amplification methods we fit separate linear models to the amplified versus nonamplified comparisons for each amplification method. The number of differentially bound spots between the two samples was calculated across the entire range of naive *P*-value thresholds from 0 to 1, at 0.00001 unit resolution. A similar analysis was used to investigate hybridization order, except that the models were fit simultaneously and the effect of each hybridization order was extracted separately using a contrast matrix. An identical tiling across *P*-values was used, in this case in the range 0 to 0.10 with a resolution of 0.0001. These analyses were performed in R (v2.4.1). All gene-lists presented are based on a *P*-value threshold of *P* < 0.05.

Agilent promoter microarray data preprocessing

Microarray data was scanned using the Agilent Feature Extraction Software (v9.5) and then loaded into the R statistical environment (v2.6.2) using the limma package (v2.12.0). Array data was preprocessed using variance-stabilizing normalization, as above, with 1000 iterations to generate highly robust results on these large arrays. Preprocessing employed the vsn package (v3.2.1) in the R statistical environment. The raw and preprocessed data were subjected to a series of quality control measures: all arrays were included in subsequent analyses. Significance-testing used *t*-tests to compare the antibody and control channels, followed by an Empirical Bayes' moderation of standard error (40) and a false-discovery rate adjustment for multiple-testing (41). The raw and pre-processed data have been made available in the GEO repository with accession GSE11245.

Spot annotation

The published array annotations for the 12k UHN CpG island microarray were based on the UCSC build hg17 of the human genome (38). To update these results for the more polished hg18 build, we implemented a new annotation algorithm using a BioPerl (v1.5.1) based

Perl (v5.8.8) script (42). First, the 3' and 5' sequence reads were overlapped using *bl2seq* (43) with a word-size of 7. In cases where a minimum 50 bp overlap was found the aggregated sequence was selected; otherwise the longer of the two sequence reads was chosen. Next, the sequences were aligned to the RepeatMasker filtered chromosomal sequence-reads of human genome build hg18 (44) using a locally compiled (*gcc* v4.1.1; *AIX* 5.2.0.0) NCBI *BLASTN* executable with a word-size of 7 (45). The best matching hit was selected as the most likely region of hybridization, and the ratio of the lengths of the top two hits was used as a measure of the likelihood of cross-hybridization. The Agilent default annotations were used for the oligonucleotide validation experiment.

RESULTS

Our analysis of ChIP-chip design parameters was conducted in the context of the genomic-binding of the well characterized Myc oncogene in an acute myelogenous leukemia cell line, HL60 (46). Optimization experiments utilized 12k CpG island microarrays which contain 12 192 genomic locations, representing 5411 distinct genomic loci (38). Validation experiments were performed using the Agilent human promoter whole genome array set.

Antibody selection, array batch, reciprocal labeling and inter-day processing

Our first series of experiments focused on assessing four key parameters of the ChIP-chip assay. Antibody selection was considered first because the immunoprecipitation step provides the starting material for the array analyses, and as such is a major determinant of the success of ChIP-chip studies. Second, we addressed the effect of array batch. This can be a major confounding variable because DNA spotted to a microarray slide may degrade or otherwise lose sensitivity, and even arrays printed in different print-runs from a single spotter can show variability. Third, we evaluated the bias introduced during the process of sample labeling. This bias refers to intensity differences between identical samples labeled with different dyes and can arise in several ways, including differential incorporation efficiencies for the two dyes, fluorophore degradation, as well as photo-bleaching rates or scanner-induced bias. Fourth, we determined whether day-to-day variations in atmospheric ozone, technical handling, scanner calibration or other, as yet unidentified, confounders affect the hybridization step.

To evaluate the effect of these ChIP-chip parameters, we conducted a saturated, fully blocked experiment (Figure 1). First, 12 independent biological replicates of

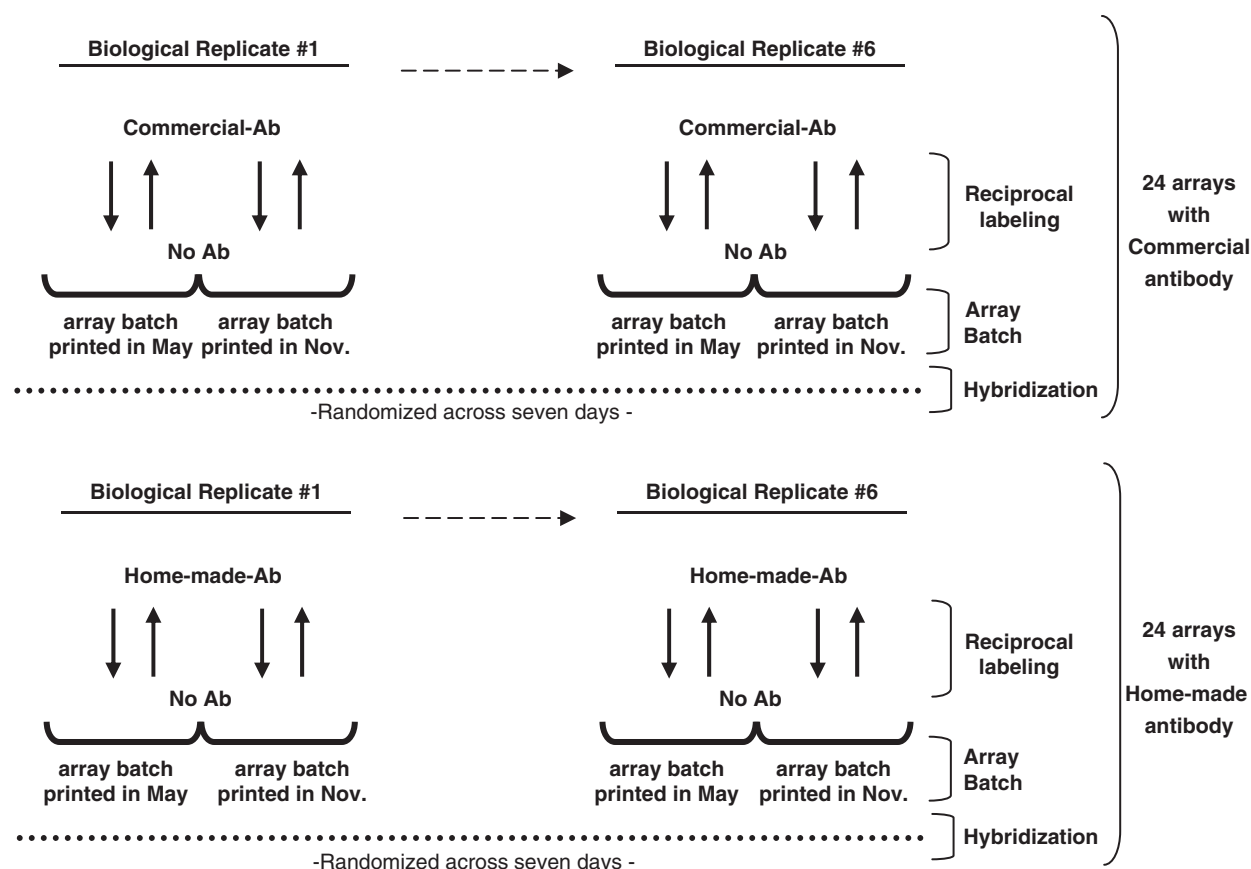


Figure 1. Experimental design to evaluate antibody effect, array batch, reciprocal labeling and hybridization. Schematic representation of the experimental design in which six independent biological replicates of cross-linked HL60 cells were subjected to ChIP with either commercial or home-made antibodies raised against the N-terminal 262 residues of the Myc oncoprotein. Each sample was hybridized to individual arrays with its matched No Ab control. Dashed arrows indicate biological replicates #2 to #5, not included in the figure for simplicity. Black arrows indicate reciprocal labeling. Dotted lines indicate the period of seven days in which the 48 arrays were hybridized.

cross-linked HL60 cells were subjected to ChIP with polyclonal antibodies raised against the N-terminal 262 residues of the Myc oncoprotein. Six of the twelve replicates were ChIPed using a commercial antibody and the remaining six using a home-made antibody raised against the identical region. For each of these twelve replicate ChIP reaction we probed two batches of arrays separated in print-age by about 6 months. This allows us to evaluate the array batch effect. Each sample was hybridized twice to each batch with reciprocal labeling. Thus, each biological replicate was hybridized to four separate arrays, leading to 24 arrays for each antibody and 48 arrays in total. Finally, these 48 arrays were hybridized in batches over 7 days, allowing us to robustly estimate the effect of date-of-hybridization (Figure 1).

To quantify the importance of antibody selection, we compared two polyclonal antibodies raised against the same N-terminal region of the Myc protein. One antibody was purchased from a commercial vendor while the other was locally produced (Figure 1). Across the 48 arrays of our study, we observed a significant

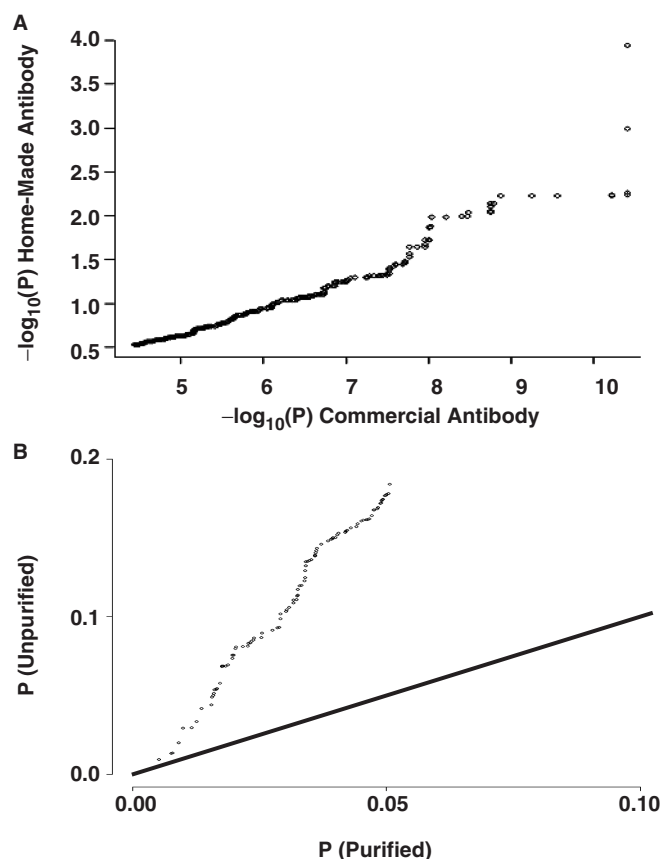


Figure 2. Measuring antibody effect. (A) Comparison of ChIP-chip results using a commercial and home-made polyclonal antibody raised against the same portion of Myc protein showed a similar linear trend and ordering by the two antibodies ($P < 2.2 \times 10^{-16}$; Wilcoxon Rank Test), however, they differed by orders of magnitude in sensitivity (axis scales). (B) Purification of the home-made antibody preparation improved sensitivity. The line represents equal sensitivity ($y = x$). Note that orders of magnitude on the axes differ between parts A and B because the replicate numbers are different (A, $n = 24$; B, $n = 4$).

antibody effect. When the P -values of the top-ranked spots on the array are compared for the two antibodies, a linear trend is observed, suggesting specificity is comparable (Figure 2A). However, despite equivalent replicate numbers and full-blocking of other parameters, as described below, P -values are distinctly lower for the commercial antibody, suggesting that it results in better signal to noise characteristics. To increase the specific activity of our home-made antibody, we subjected it to a purification procedure to remove contaminating serum proteins, and compared purified and unpurified home-made antibody in a similar fashion (Figure 2B). A linear trend was again observed, with the purified antibody showing lower P -values than the unpurified. Note that the magnitude of P -values in Figure 2A and B are different because of the different replicate numbers involved (Figure 2A, $n = 24$; B, $n = 4$ for each antibody). These data suggest that antibody purity is a key factor influencing the specificity and sensitivity of ChIP-chip studies and may improve the performance of some antibodies.

To evaluate the effect of array batch, dye bias and inter-day hybridization, we fit spot-wise linear models to the results of each parameter separately and plotted the Gaussian densities of the magnitudes of these effects (Figure 3). These Gaussian densities are essentially smoothed histograms. Their height (y-axis) reflects the number of spots on the arrays affected by each parameter at the given fold-change (x-axis). Each curve reflects a different source of variability (dye, batch and date). The numbers under the plot give the number of spots affected by each parameter at a given fold-change threshold. All three curves are unimodal, with a sharp peak at the origin. This shows that the majority of spots on the array are unaffected by each parameter. Of the spots that are affected, the magnitude is generally very modest, in the range of -0.5 to $+0.5 \log_2$ units. This corresponds to an

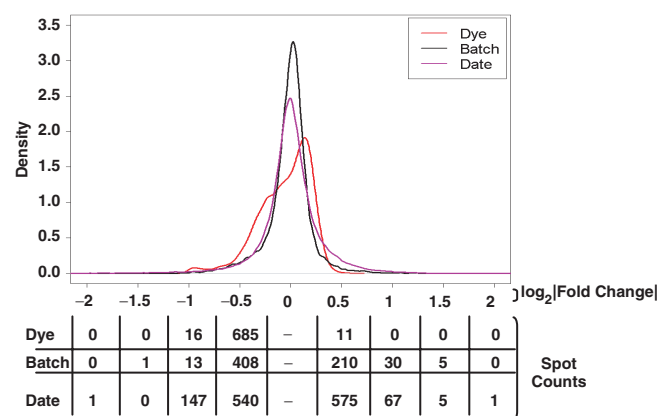


Figure 3. Evaluating array batch effects, dye-bias and inter-day processing. To assess the effects of array batch, dye-swap, and hybridization date we fitted separate spot-wise linear models to the data from our study for each of these three parameters. Gaussian kernel densities were then plotted for the coefficients of the fits for each model. Each curve is unimodal, with a sharp peak around the origin, indicating that most spots on the array do not vary with these design parameters. The spot counts along the bottom of the figure further quantify the degree of variability at several thresholds ($0.5 = 1.4$ -fold, $1.0 = 2.0$ -fold, $1.5 = 2.8$ -fold, $2.0 = 4.0$ -fold).

effect of 1.4-fold. Overall, no >10% of spots on the array show large variability towards any of these parameters—suggesting that both the ChIP protocol and the CpG island arrays used are robust towards perturbations in any of these factors.

Despite this basal similarity there are differences across the three parameters. The curve of dye-bias (red) shows significant asymmetry, corresponding to a consistent Cy3 bias. While only 11 spots show Cy5/Cy3 ratios of 1.4-fold or higher, 685 spots show Cy3/Cy5 ratios of 1.4-fold or greater. This marked asymmetry highlights the importance of balancing experiments across the two dyes used in the study.

The effect of array batch shows a similar, but smaller asymmetry. Older arrays consistently show lower signal intensities than newer arrays, with 408 spots showing an old/new ratio of at least 1.4-fold, while only 210 spots show a new/old ratio of similar magnitude. This variability suggests that experiments should be conducted with arrays of a single batch, or experimental samples randomized across multiple batches.

Surprisingly, the effects of hybridization date affect almost 10% of the spots on the array, despite the same, experienced hands conducting all hybridizations in this series of experiments. This effect is symmetrical, with approximately equal numbers of spots showing date-dependent enrichment and depletion. The source of this bias is unknown. It is possible that atmospheric ozone may have differed from day-to-day. We and others have experienced Cy5 squelching due to ozone (47,48). However, in this case ozone is not likely to be the source of the bias, as we chose to conduct these experiments during times of consistent and low atmospheric ozone. Variations in the efficiencies of the labeling reaction and subsequent column-clean-up may account for this effect. It is also possible that variation in handling at the level of post-hybridization washes may contribute to this inter-day variability. Another possibility is that scanner calibration varied across the course of our experiment, but the biases described here are not temporally-dependent (data not shown). This suggests that if scanner-calibration is an issue, then it is a random drift rather than a progressive degradation.

As an alternative visualization of this data, we provide histograms of each factor as Supplementary Figure 1. In addition, the raw and normalized data are available in the GEO repository, while the linear model fits are available as Supplementary Tables 1–3.

Amplification method

Sufficient DNA for microarray hybridization cannot usually be obtained from a single ChIP reaction. The amount required can be achieved by pooling multiple ChIP reactions (12), but this approach is expensive and labor intensive. Further, when dealing with biologically limited samples, such as primary patient material, repeated ChIPs may not be possible. Indeed in such cases pooling multiple ChIPs may introduce intra-sample heterogeneity (49). To overcome this rate-limiting step, the precipitated DNA in a single ChIP reaction is usually

amplified. A variety of PCR-based methods exists, including LM-PCR, WGA, random primed PCR (RP-PCR) and T7 primed PCR methods.

We develop an assay to evaluate the bias introduced by amplification protocols. For each amplification protocol we take a pool of total genomic DNA and amplify one aliquot. We then hybridize equal amounts of amplified and unamplified DNA on a microarray slide. This procedure is repeated to determine the number and nature of the spots showing amplification bias (Figure 4A). Using this method we tested the three most common amplification techniques employed in ChIP-chip experiments, RP-PCR, WGA and LM-PCR (Figure 4A). To consider parameter sensitivity for the LM-PCR method we evaluated protocols with low (LM-PCR A, 45 cycles) and high (LM-PCR B, 60 cycles) PCR cycle number.

For each amplification method, we considered the fraction of spots on the array that showed altered hybridization between amplified and unamplified samples as a function of the *p*-value threshold (Figure 4B). Methods with reduced amplification bias will show lower curves, especially at experimentally relevant lower *P*-value thresholds (Figure 4B, inset). The LM-PCR A and WGA amplification methods alter the hybridization of many fewer spots than either RP-PCR or LM-PCR B. These data demonstrate a practical and affordable method of evaluating a variety of amplification protocols using genomic DNA.

Hybridization control

A variety of different hybridization controls have been used for ChIP-chip studies. For example, some groups have employed a ‘no antibody’ control (12), while others have used total genomic DNA (11,50). To determine if there are significant differences between these hybridization controls, we designed a series of experiments to test each approach (Figure 5A). Specifically, we compared direct hybridization of ChIP samples against a mock treated control (Direct No Ab) and against an IgG antibody control (Direct IgG). We also considered the use of total input DNA as a ‘denominator’ by hybridizing ChIP and IgG samples separately against total-input DNA (Indirect No Ab), then comparing their intensities indirectly during statistical analysis by using a linear model. The results (Figure 5B) are surprisingly invariant from one control to the next, and at any given *P*-value the number of hits identified in each experiment is nearly identical. Nevertheless, the two direct designs show more sensitivity than the indirect approach in a threshold-independent manner.

Validation studies

To evaluate the optimized parameters determined above, we conducted a new Myc ChIP-chip experiment with them. Thus, we used a purified antibody for the ChIP reaction, DNA was amplified by LM-PCR, we hybridized a single CpG island array batch from a recent print run, and IgG was used as the hybridization control. Dye swaps were randomized across hybridization dates, and the entire experiment was conducted at high

replication ($n = 13$). By using this highly optimized design, we expected to both minimize bias and increase specificity. Following preprocessing and statistical analysis of the raw data we obtained a list of 534 Myc bound spots (Supplementary Table 4). This represents $\sim 9.9\%$ of the

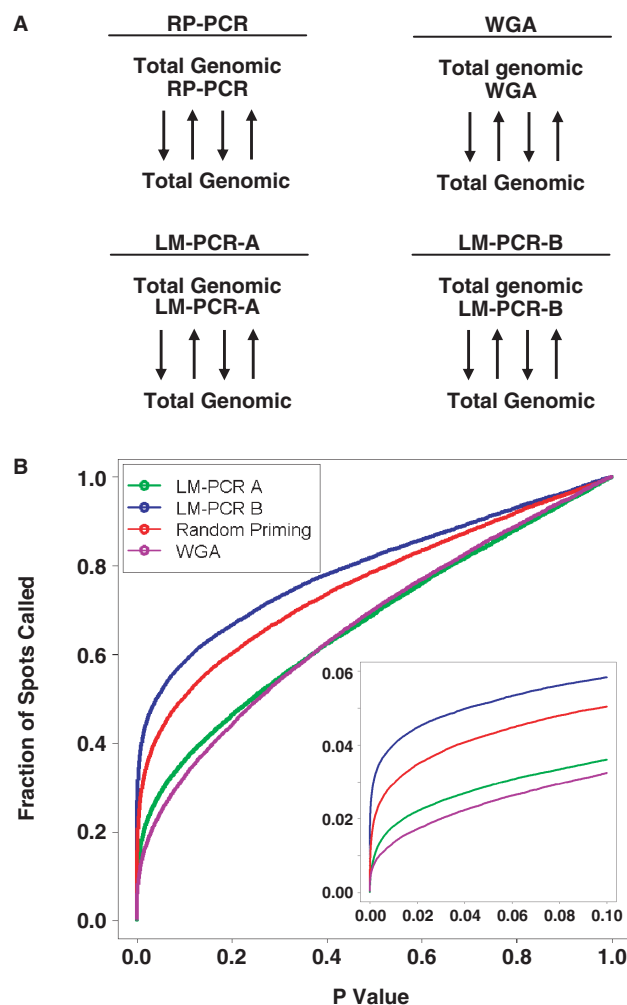


Figure 4. Comparing DNA amplification methods. To evaluate whether specific DNA amplification protocols introduce different degrees of bias, we surveyed the genome-wide effects of three amplification protocols. (A) Total genomic DNA was amplified with one of four different protocols: random priming (RP-PCR), WGA and two LM-PCR protocols (LM-PCR-A and LM-PCR-B). The resulting DNA was then hybridized to a CpG Island microarray against an equal amount of unamplified total genomic DNA. This procedure was repeated four times for each of the three amplification methods, and the raw data was pre-processed with VSN and model-based t -tests (see Materials and methods section). Black arrows indicate reciprocal labeling. (B) The fraction of spots on the array that are differentially amplified (fraction of spots called) is plotted against the P -value threshold for each amplification method. The y -axis gives the fraction of spots differentially amplified, thus a value of 0.1 indicates that 10% of the spots on the array have been affected by the amplification protocol. The $y = x$ line reflects the fraction of spots that would be expected to be altered by amplification by chance alone. All four curves lie above this line, demonstrating the large effect of amplification. The curves for LM-PCR A and the WGA lie below that of the other two methods for all P -values, demonstrating that these two methods introduce the smallest amplification bias amongst the four protocols tested. The inset provides a zoom-in of the P -value range from 0 to 0.1, where thresholds for most experiments are selected.

5411 distinct genomic loci represented on this array platform (38). Importantly, 35 of these 535 spots correspond to known Myc targets annotated in the Myc target gene database (51), providing strong support for the validity of this gene list.

To further generalize our results, we then performed a second validation experiment using a different microarray platform—Agilent genome-wide oligonucleotide promoter arrays. We again employed our optimized parameters, using a purified antibody, a single batch of arrays and IgG as a hybridization control. For this second validation, we used the WGA method to amplify the DNA. As these oligonucleotides are a relatively new platform we rigorously evaluated array quality and found no evidence of spatial or distributional artifacts (data not shown). Following preprocessing and statistical analysis we obtained a list of 53 453 genomic regions that bind c-Myc, corresponding to 10 196 unique genes or genomic features (Supplementary Tables 5 and 6). This represents 11.1% (53 453 of 481 909) of the unique genomic regions and 28.9% of the unique genes represented on the array, indicating wide-spread Myc binding. On average each unique gene was represented by 5.24 probes, giving a high-degree of internal replication within the array.

Comparison of the CpG island and promoter arrays is confounded by the lack of perfect correspondence between the genomic features represented on each array. For example, the CpG island arrays might interrogate a region downstream of the transcriptional start-site,

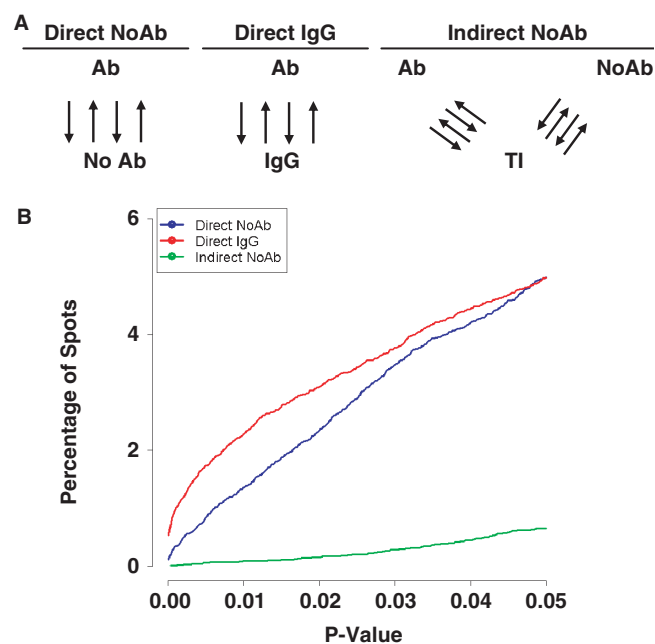


Figure 5. Comparing hybridization controls. A number of potential control samples can be used for ChIP-chip studies. (A) Three common approaches were compared, including two direct hybridization approaches (No Ab and IgG) and an indirect hybridization approach (No Ab). For each approach ChIP-chip studies of Myc in HL60 cells were performed. Black arrows indicate reciprocal labeling. (B) The sensitivity of each approach was plotted as the percentage of spots identified as differentially bound versus the P -value. While the three methods displayed similar performance, the two direct methods show higher sensitivity than the indirect method.

while the promoter array interrogates one upstream of the start-site. Nevertheless, we compared the hits from the CpG island array that were within 10kb of an annotated Entrez Gene transcriptional-start site to those from the promoter array. Fully, 81% (208/256) were confirmed on the promoter arrays at a 10% FDR. Some fraction of the unconfirmed cases are attributable to differences in the genomic regions interrogated, indicating that inter-platform variability is at most 19% in this study.

Surprised at the very large fraction of promoter regions that exhibited c-Myc binding in our genome-wide analysis,

we sought to validate these results using gold-standard Q-PCR on independent biological replicates. We selected 50 random genomic loci from our gene-lists (Supplementary Tables 4–6) and primer pairs to interrogate them by Q-PCR. We used three Myc ChIP biological replicates in HL60 cells that were completely separate from any of the samples used in either the optimization or validation studies. As a negative control we employed a region of chromosome 6 that showed no evidence of Myc binding in the ENCODE study. Our Q-PCR results (Figure 6 and Supplementary Table 7) verify the extensive binding of

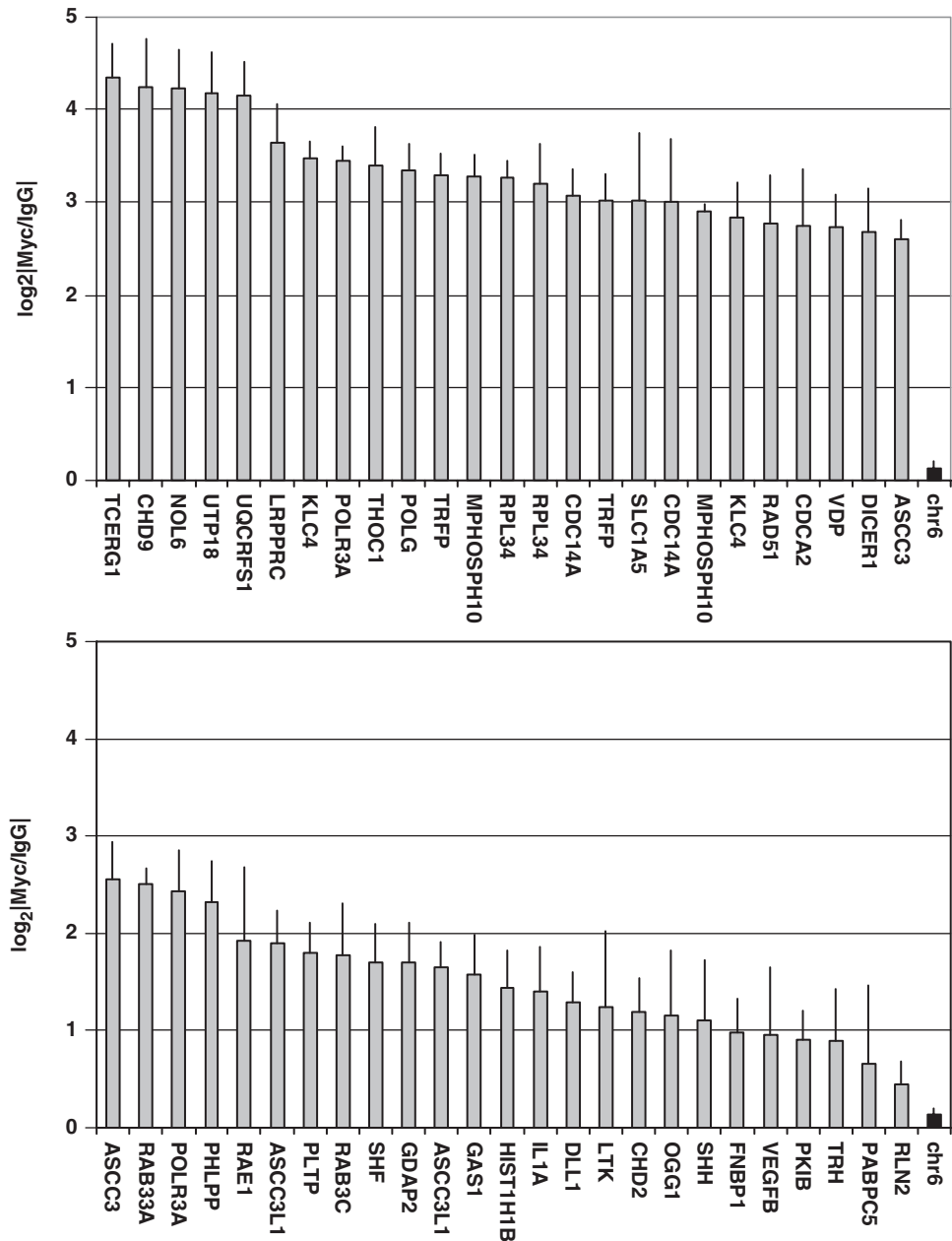


Figure 6. Myc recruitment to regulatory regions of novel target genes in growing HL60 cells. ChIP assays with antibody to Myc and IgG control antibody from exponentially growing HL60 cells. DNA was Q-PCR amplified by using primers against 50 distinct genomic regions. A negative control region on chromosome six that showed no Myc binding in any cell-line of the ENCODE study was also included and is shown twice for reference purposes only. This region is shaded in black and is labeled 'chr6'. Each column represents a specific region, identified by the HGNC gene-symbol. When two different regions of the same gene were tested, the same gene can be found twice in the figure (Supplementary Table 8). The y-axis gives the mean $\log_2[\text{Myc/IgG}]$ values for each genomic region. Error bars represent SDs.

c-Myc in HL60 cells, with an overall validation rate of 45/50. Further, the array and PCR results are highly correlated on a region-by-region basis, both in terms of statistical significance (Spearman's $\rho = 0.533$, $P = 7.80 \times 10^{-5}$) and magnitude of enrichment (Spearman's $\rho = 0.598$, $P = 5.77 \times 10^{-6}$). These data show that our optimized ChIP-chip experimental parameters allowed us to identify novel *bona fide* Myc target genes using two separate microarray platforms as well as quantitative PCR.

DISCUSSION

ChIP-chip is a relatively new technology that has quickly become a standard method for screening protein–DNA interactions *in vivo*. However, it is also a highly complex method, with many tunable parameters. We set out to systematically test a large subset of these experimental design parameters. Many previous studies of microarray reproducibility have employed spike-in or mixture designs (23,31,33,35), but it is unclear if spike-in or mixture experiments reproduce the underlying complexity of real biological samples. Accordingly, we chose to focus on a biologically well-characterized system: involving the Myc oncogene as a regulator of gene transcription in the HL60 human myeloid leukemia cells (12).

The first experimental design parameter considered was the influence of different antibodies on the results of a ChIP-chip study. Intuitively, the nature of the antibody is a limiting step for ChIP-chip studies. To put a lower bound on the variability introduced by different antibodies we selected two polyclonal antibodies raised against the same portion of the Myc protein (the N-terminal 262 residues, N262) but derived from different sources. Critically, the two antibodies showed a broadly linear, well-ordered trend ($P < 2.2 \times 10^{-16}$; Wilcoxon Rank Test), but differed significantly in sensitivity (Figure 2A). We subjected the lower sensitivity antibody to a column purification procedure to remove contaminating serum proteins, and this purification procedure significantly improved performance (Figure 2B). Clearly, antibody selection is a critical element of ChIP-chip studies, and antibody-specific activity is an important parameter to consider prior to hybridization.

Next, we considered the effects of inter-batch variability of the CpG island microarrays. This array platform has been widely used by other groups (27,50,52), giving confidence in its reliability. We tested the variability between two batches of arrays and observed a consistent bias in signal-intensity, with the older array batch showing lower signal (Figure 3). This bias is highly reproducible, as we analyzed 24 arrays from each batch. The bias is symmetric, and therefore can lead to both false positives and false-negatives. Fortunately, straightforward methods exist to control this bias. First, whenever possible, arrays from a single batch should be used. Second, when multiple batches are used samples should be randomized across batches. Third, linear-modeling methods can be used to explicitly account for and remove batch variability. The first two methods are standard experimental design

practices, while the third has been successfully used in the analysis of mRNA expression array data (53).

Third, we considered the effects of reciprocal labeling (dye-swapping) a single sample with the Cy3 and Cy5 dyes. This topic has been extensively studied from both theoretical (54,55), and empirical (56) perspectives. These studies found small, but consistent effects of reciprocal labeling. While reciprocal labeling has been shown to have less experimental value than true biological replicates, they appear to carry more information than simple repeated measures (56). Very moderate effects of reciprocal labeling were observed, affecting only about 0.1% of the spots on the array at a two-fold change level (Figure 3). There are many more spots affected by dye status at a less stringent 1.4-fold change threshold, however changes of this magnitude introduce a much smaller risk of altering the spot-wise statistical analyses. This indicates that, at least for ChIP-chip with our CpG island arrays, reciprocal labeling is not necessary and dye-bias is a negligible confounder. Nevertheless, it seems prudent to randomize samples across dye-status to account for unanticipated dye-bias in specific experimental scenarios, especially because of the profound asymmetry in this parameter.

The fourth experimental parameter considered was the effect of hybridization date. Because our experiment was fully blocked across all factors, we were able to directly estimate the effects of this parameter with a linear model. We were surprised to find that the day on which hybridization was performed had a greater influence on the final results than either the batch of arrays or the use of dye-swaps (Figure 3). The sources of this bias are unclear. The symmetrical distribution of the effect suggests that neither ozone-status nor scanner settings are a major factor, suggesting random drift within the scanner. Perhaps use of a mechanical hybridization and wash station would decrease inter-day variability, although this variability may also result from the labeling reaction or the subsequent column clean-up procedure. Control of this factor is straightforward: samples should be randomized across experimental day, so that biological replicates of a given condition are hybridized on different days.

The fifth experimental parameter considered was the method of DNA amplification. While the antibody-based ChIP protocol imparts specificity and sensitivity to ChIP-chip experiments, the quantity of DNA necessary for hybridization cannot usually be obtained in a single ChIP. Two basic strategies have evolved to cope with this issue: pooling of multiple ChIPs (12) and amplification of ChIP DNA (26). A variety of different PCR-based amplification methods have been developed and are in use by different groups. Assessing the performance of these methods has been challenging. To directly compare an amplified ChIP sample to an unamplified ChIP sample requires the pooling of a large number of ChIPs for each replicate, resulting in significant costs, time and labor. Further, care must be taken in the experimental design to ensure that the reduction in variance that results from pooling ChIP reactions does not confound the effects of the amplification procedure. As a result

of these financial and technical factors, only one study has assessed the performance of amplification methods for ChIP-chip data (23), although several such studies do exist for mRNA experiments (57,58) where a much larger amount of starting material is readily available. To resolve this issue, we have developed a novel approach that hybridizes amplified against non-amplified genomic DNA (Figure 4A). We evaluated four amplification protocols: RP-PCR, WGA and two variations of LM-PCR. Because of its simplicity, this procedure could be readily repeated and an $n = 4$ was generated for each amplification procedure. The number of spots found to be differentially hybridized between the matched amplified and unamplified samples is a measure of amplification bias (Figure 4B). Interestingly, the performance of random-priming falls in between that of the two LM-PCR protocols, indicating that specific parameter choices within a protocol are important for determining overall performance. The WGA and low-cycle LM-PCR protocols showed similar performance, suggesting that one of these methods should be selected to minimize amplification bias. Our method of comparing amplification procedures provides a new way to measure variability, independent of antibody or biological question, at a reasonable cost.

The final experimental design parameter considered was the selection of a negative control for hybridization to the array. We considered three approaches (Figure 5A), including two direct and one indirect method. For each method we performed four Myc ChIP-chip experiments in HL60 cells. When we compared the sensitivity of these methods (Figure 5B) the two direct methods performed comparably, with no significant differences between the IgG and no-antibody controls. The indirect design, however, did show reduced sensitivity at any given P -value. Thus, direct designs, which use half as many arrays as indirect designs, are favored for both cost and sensitivity.

Having extensively optimized these six parameters, we set up two Myc ChIP-chip experiments to test our optimization in an integrated way. Our first experiment used extensive biological replication ($n = 13$) on CpG Island arrays, while our second used lesser replication ($n = 5$) on much larger whole-genome oligonucleotide promoter arrays. To validate the results of these two studies we employed Q-PCR using biologically independent samples. Of the 50 unique primer sets we analyzed, 45 were validated, for a success rate of 90%. This strong evidence that the vast majority of the 10 196 genomic locations found to be associated with Myc in HL60 cells are true positives. Importantly, we showed that the validation rate remained high in the bottom portion of our list. This suggests that the statistical analysis was conservative, and that additional hits might be uncovered by relaxing our P -value threshold from 0.05 to 0.10 or higher. This is particularly important because many published ChIP-chip studies use a P -value threshold of 0.001, 50 times more stringent than used here (11). Overall, our extensive validation confirms the success of our parameter optimizations.

Over the past decade, a series of studies from multiple groups characterized the major experimental design

parameters for mRNA expression studies (33,56,59–61). A common theme of these studies is that it is necessary to use both empirical and statistical methodologies to optimize the experimental design. We have provided the first comprehensive overview of experimental design parameters for ChIP-chip data, testing both parameters related to all microarray experiments and those unique to ChIP-chip studies. We find good concordance of ChIP-chip and mRNA expression design characteristics. For example, in both cases, indirect hybridization designs appear to be more costly without providing a significant advantage over simpler direct designs. Similarly, we have shown that biological replicates are more important than dye-swaps, just as identified for mRNA expression arrays (56). We also demonstrated that antibody characteristics and amplification methods are major sources of variability in ChIP-chip studies. For example, using an antibody of maximal specific activity appears to be critical for increasing the sensitivity of ChIP-chip studies. In addition, we introduced a novel method for testing amplification bias in an antibody-independent manner. This study indicated that amplification procedures can significantly bias ChIP-chip results, and provides a simple, efficient method for benchmarking the bias of specific amplification protocols.

Our findings also raise a number of intriguing questions surrounding the antibody and amplification results. We demonstrate that antibody-specific baseline variability exists in ChIP-chip studies, and that while it can be mitigated it cannot be completely removed. It would be of great interest to replicate these studies with monoclonal antibodies, or polyclonal antibodies raised against different regions of a protein. In this manner, it might be possible to identify characteristics that predict which antibodies will be most sensitive or specific in a ChIP-chip study. Alternatively, this may not be a generalizable parameter and may be protein-specific. Similarly, we have presented a novel technique for studying amplification bias, and identified both a variant of LM-PCR and the WGA protocol as most favorable in this regard. Nevertheless, all amplification protocols tested here introduced a significant amount of bias. Our strategy for bias-identification should prove useful in the development of reduced-bias amplification protocols.

All of our analyses were performed studying the c-Myc transcription factor in the HL60 cell line. While this results in a consistent comparison across our many experiments, this also means that our results are formally restricted to a single biological system. However it is highly probable that our findings can be widely generalized. First, our validation experiment used a separate microarray platform with widely different feature sizes and again found a high confirmation rate by Q-PCR. Second, several of the parameters studied here are common to many microarray studies, including the choice of amplification method and the inter-batch and inter-day variability of microarrays and scanners. In particular, our analysis of amplification is relevant to studies of genomic variation using array-CGH and SNP-arrays. Third, the quality of antibody used in ChIP reactions will affect the quality of data retrieved by

microarray, PCR and sequencing-based techniques. Thus we expect these findings to be broadly applicable to many fields.

As ChIP-chip studies continue to broaden in the scope and detail of the biological questions they probe, the application of optimized and validated experimental designs will be a significant advantage to the field.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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